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The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*

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Abstract The kinetics of nisin production was studied in batch cultures using a construct of *Lactococcus lactis* subsp. *lactis* C2SmPrt$^-$, containing a transposon (Tn*Nip*) that encodes nisin production. The introduction of Tn*Nip* into C2SmPrt$^-$ significantly lowered the specific growth rate and the maximum A_{620} reached was reduced from 15.2 to 11.0. The effect of nisin concentration and nutrient depletion on nisin production of the construct, C2SmPrt$^-$(Tn*Nip*), was examined. Nisin production was found to be inhibited by high concentrations of nisin, when grown in excess nutrient, even though growth of the culture continued because nutrient limitation was not operating. However, in low nutrient concentrations nisin production was limited by nutrient depletion. The specific growth rate of C2SmPrt$^-$(Tn*Nip*) was altered, by using different nutrient concentrations and different sugars, in order to examine the relationship between nisin production and growth. Nisin production was shown to be growth-associated for most of growth, but near the end of growth, when the specific growth rate was 0.05 h^{-1} or less, the production ceased.

Introduction

Nisin belongs to a group of bacteriocins called lantibiotics. Lantibiotics are bactericidal peptides and are characterized by the presence of an unusual amino acid, lanthionine. Nisin has strong antimicrobial activity against certain gram-positive bacteria and is used commercially in food preservation (Hurst 1981). The genes involved in nisin biosynthesis, immunity and control are grouped into a contiguous cluster (Siegers and Entian 1995). The *nisA* gene is the structural gene encoding the peptide nisin. This gene is followed by genes *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, *nisK*, *nisF*, *nisE* and *nisG*. The genes *nisB* and *nisC* encode products that are involved in maturation of the prepeptide (Engelke et al. 1992). The gene *nisT* is involved in transport (Engelke et al. 1992), *nisI*, *nisF*, *nisE* and *nisG* are involved in immunity to nisin (Engelke et al. 1994; Siegers and Entian 1995) and *nisP* is involved in processing of the secreted prepeptide (van der Meer et al. 1993). The regulation of nisin biosynthesis is mediated by the two regulatory genes *nisR* and *nisK*, which encode the response regulator (NisR) and the histidine kinase sensor (NisK) respectively (Engelke et al. 1994). NisK recognizes an external signal and transmits this signal by phosphorylation of NisR, which in its phosphorylated form stimulates the transcriptional initiation at the nisin promoters (Qiao et al. 1996). The mature peptide nisin is the external signal, and therefore nisin biosynthesis is autoregulated by its own product.

The nisin-producing lactococcal strains are fastidious organisms that require rich media, such as milk or a complex organic medium, for good growth. Several workers have attempted to develop an optimal medium for maximal nisin production. They have concluded that maximum nisin production is directly related to biomass formation and production is closely associated with growth (Hirsch 1951; Egorov et al. 1971; Kozak and Dobrzanski 1977). In addition, production has been shown to stop completely when cells enter the stationary phase (De Vuyst and Vandamme 1992). Nisin is therefore regarded as a primary metabolite. The growth medium used for nisin production is basically similar to that used for growth of starter cultures, i.e., medium that supports high growth rate and high biomass formation.

Increasing the phosphate concentration to 5%, using KH_2PO_4, stimulated nisin production, and a complex medium supplemented with a nitrogen source also gave very high nisin yields (De Vuyst and Vandamme 1993). Nisin biosynthesis is strongly dependent on the presence of a sulphur source, and the amino acids serine, threo-

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nine and cysteine stimulated nisin production without affecting the final cell yield (De Vuyst 1995). Nisin production has also been studied in continuous culture, in which it was found that nisin biosynthesis increased with increasing lactose concentration, but that, at high lactose concentrations, nisin production declined (Meghrous et al. 1992). Continuous production of nisin in a bioreactor system coupled to a microfiltration module has been described (Taniguchi et al. 1994). It was found that nisin productivity with respect to both cultivation time and the quantity of glucose consumed was enhanced by maintaining a low concentration of lactic acid in the broth.

To date, studies on the kinetics of nisin production have addressed some factors that affect the level of nisin produced. In this communication, control aspects of nisin production were investigated. It reports product inhibition of nisin production and the specific growth rate at which nisin production ceases. It also reports the effect on growth kinetics of the producer organism following introduction of a transposon that encodes nisin production.

Materials and methods

Bacterial strains and media

The nisin-producing strain used as the donor in the conjugation experiment was *Lactococcus lactis* subsp. *lactis* (*L. lactis*) ATCC 11454. This strain contains a conjugative transposon (Tn*Nip*), which encodes nisin production and resistance and sucrose utilization. The recipient was *L. lactis* C2 (Commonwealth Scientific & Industrial Research Organization, Australia). *Micrococcus luteus* (ATCC 9341) was used as the indicator organism in the nisin assay. Growth responses on plates were determined using the following media: lactococcal strains were grown on M17 (Terzaghi and Sandine 1975) supplemented with 0.5% (w/v) glucose (M17G), sucrose (M17S) or lactose (M17L); proteinase-positive (Prt^+)and proteinase-negative (Prt^-) strains were differentiated on fast slow differential agar (FSDA) (Huggins and Sandine 1983). The lactococcal strains were maintained as previously described (Terzaghi and Sandine 1975). Cultivations were carried out using a medium containing nutrient (1.5 N) and 60 g glucose l^{-1}, unless stated otherwise. This medium was prepared as follows: 3.0 N was made by using polypeptone peptone (21 g), beef extract (10.5 g), yeast extract (5.25 g) and $MgSO_4 \cdot 7H_2O$ (0.525 g) in 490 ml water and autoclaving; L-ascorbic acid (1.05 g) dissolved in 10 ml water and sterilized by filtration was then added; glucose (60 g) was dissolved in 500 ml water and autoclaved and the two preparations were then mixed together to give the required nutrient and glucose concentrations.

Microbiological procedures

Elimination of a plasmid that encodes proteinase production was carried out as previously described (Orberg and Sandine 1985). Streptomycin-resistant mutants were isolated as follows. A 5.0-ml culture of $C2Prt^-$ was grown overnight, and the cells were collected and resuspended in 5.0 ml saline. Aliquots (0.2 ml) were spread-plated onto M17G containing 500 μg streptomycin ml^{-1} and incubated at 30 °C for 2 days. Spontaneously arising mutants were purified on the same medium. The conjugation experiment was performed using the filter mating method with α-chymotrypsin (Steele and McKay 1986). The transconjugants were selected on M17S containing 500 μg streptomycin ml^{-1}. Plasmid DNA was isolated as previously described (Anderson and McKay 1983). Agarose gel for electrophoresis was prepared from 0.6% (w/v) agarose and tris base (40 mM), acetic acid (20 mM) and EDTA (20 mM) buffer. Gels were run at 80 V for 5 h.

Inoculum propagation and cultivation

The strains to be cultivated were streaked-out from a stock onto an appropriate selection plate and incubated overnight at 30 °C. A single colony was then used to inoculate 20 ml cultivation medium, which was incubated overnight at 30 °C. The 20 ml was then inoculated into a standard bioreactor and grown at 30 °C. The culture was harvested at exponential phase and was used to inoculate the test bioreactors. The size of the inoculum was calculated so as to give an initial A_{620} reading of approximately 1.0, unless stated otherwise. The cultivations were conducted as anaerobic batch cultures in 500 ml Quickfit-type bioreactors. The pH was maintained at 6.0 and the temperature at 30 °C. The anaerobic conditions were maintained by filling the head space with a mixture of N_2 and CO_2 gases flowing at 150 cm^2 min^{-1}. All cultivations were done in triplicate and representative results are shown. In one experiment, purified nisin was added to the cultivations. The purification was done by dissolving Nisaplin (Aplin and Barrett) in 0.02 M HCl and removing solids by centrifugation at 5000 rpm for 10 min.

Absorbance measurement and nisin assay

Samples (0.2–0.5 ml) were collected hourly for absorbance determination. They were appropriately diluted with dispersant then allowed to stand for 15 min before determining A_{620}. Samples of 4 ml were also collected for nisin assay at the same time as the absorbance samples. They were adjusted to give a pH of 2.0 ± 0.5 with a few drops of concentrated HCl and stored at −20 °C until all samples were collected; the nisin assays were then carried out. The samples were thawed, heated in a boiling water bath for 10 min and then cooled to room temperature. They were appropriately diluted in 0.02 M HCl and nisin assays were performed by an agar-diffusion method (Tramer and Fowler 1964). All assays were performed in triplicate and averaged results are shown. Nisin concentrations are stated as the relative nisin concentration (RNC).

Results

Generation of a nisin-producing construct of *L. lactis* C2

The nisin-producing construct generated for this study required three steps. First, the proteinase (Prt) function was eliminated from *L. lactis* C2 by removing the Prt plasmid, because the encoded proteinase is known to degrade nisin in that host (Kim et al. 1990). Cured clones were selected on FSDA plates. Prt^+ colonies were large and yellow, whereas Prt^- colonies were small and white. One Prt^- colony was purified on the same medium and plasmid-profiled. It appeared to have lost three plasmids, which were 18, 28 and 46 kb in size. The strain retained four other plasmids. This strain was designated $C2Prt^-$. In addition to being Prt^-, it was also shown to have become Lac^-. Second, spontaneous streptomycin-resistant mutants of $C2Prt^-$ were isolated, to facilitate selection in the subsequent conjugation step. One mutant was purified on the selection medium and was designated as $C2SmPrt^-$. Thirdly, Tn*Nip* was introduced

into C2SmPrt$^-$ by conjugation with *L. lactis* ATCC 11454. The frequency of transfer was 9.0×10^{-6} transconjugants/donor cell. One transconjugant was purified and the phenotypes checked. It was nisin-producing, nisin-resistant and able to utilize sucrose. The construct was designated as C2SmPrt$^-$(Tn*Nip*).

Effect of the Tn*Nip* on growth

Batch cultures were carried out using C2, C2Prt$^-$, C2SmPrt$^-$ and C2SmPrt$^-$(Tn*Nip*). The objective was to compare the growth kinetics of C2 and the three derivatives, and to examine the effect of the Tn*Nip* on growth. Other objectives were to examine the effect on growth of removing the Prt function and the introduction of a streptomycin-resistant marker on growth. The maximum absorbances (A_{max}) reached by C2, C2Prt$^-$, C2SmPrt$^-$ and C2SmPrt$^-$(Tn*Nip*) were 14.5, 16.1, 15.2 and 11.0 respectively. The maximum specific growth rates (h^{-1}) were 0.85, 0.79, 0.72 and 0.49 respectively. The growth kinetics of C2, C2Prt$^-$ and C2SmPrt$^-$ were quite similar, whereas that of C2SmPrt$^-$(Tn*Nip*) was quite different. The removal of the Prt function slightly increased the A_{max} of the host, and the introduction of the streptomycin-resistant marker seemed to have no significant effect on growth. However, the introduction of the Tn*Nip* into the host significantly lowered the specific growth rate and the A_{max} reached.

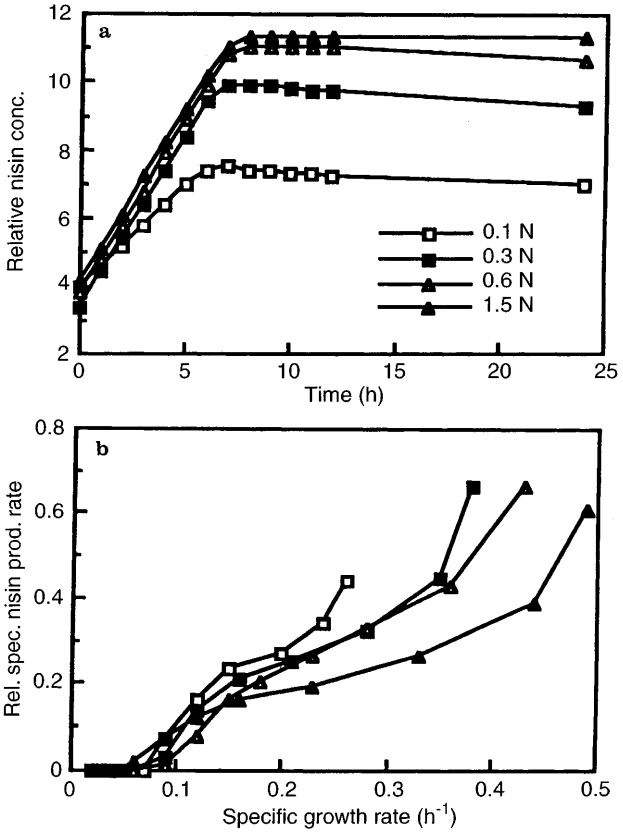

Fig. 1a,b Nisin production using different nutrient concentrations: **a** the level of nisin produced during cultivation; **b** the relationship between nisin production and growth. See Materials and methods for descriptions of media 0.1 N–1.5 N

Relationship between nisin production and growth

The relationship between nisin production and growth was investigated by altering the specific growth rate. First, the specific growth rate was altered by using different nutrient concentrations. C2SmPrt$^-$(Tn*Nip*) was cultivated on media containing four different nutrient concentrations, i.e., 0.1 N, 0.3 N, 0.6 N and 1.5 N. The level of glucose was the same for all at 60 g l^{-1}. The A_{max} values reached were 5.1, 8.0 and 10.1 and 11.0 respectively. The graph plotting the relative nisin concentration (RNC) against time (Fig. 1a) shows that the maximum RNC values obtained were 7.5, 9.9, 11.0 and 11.3 respectively. The graph of the relative specific nisin production rate against the specific growth rate (Fig. 1b) shows that nisin production was growth-associated for most of growth, but near the end of growth, when the specific growth rate was approximately 0.05 (h^{-1}), the relative specific nisin production rate was zero.

Secondly, the specific growth rate was altered by using different sugars. C2SmPrt$^-$(Tn*Nip*) was cultivated on 1.5 N containing three different sugars, i.e. glucose, sucrose or galactose at 60 g l^{-1} each. The A_{max} reached were 11.0, 13.8 and 6.2 respectively, and the maximum specific growth rates (h^{-1}) were 0.49, 0.49 and 0.23 respectively. The maximum RNC obtained were 11.3, 10.5, and 8.5 respectively. This study once again demonstrated (data not shown) that nisin production was growth-associated for most of the growth period, but near the end of growth, when the specific growth rate was approximately 0.05 (h^{-1}), the relative specific nisin production was zero.

Nisin production using different inoculum sizes

The results that have been obtained indicate that the maximum level of nisin achieved correlates with the A_{max} reached. Therefore, in this study, two larger inoculum sizes were used in addition to the standard inoculum size to see if the total amount of nisin produced could be increased if the culture grew to a higher A_{max}. Initial bioreactor absorbance were 1.0 (small), 3.6 (medium) and 6.1 (large). The A_{max} reached in the three cultivations were 11.0, 13.2 and 16.0 respectively. Despite the increases in the A_{max} there was no increase in the maximum levels of nisin, which were 11.2, 11.1 and 11.0 respectively. Irrespective of the cell concentration, nisin production closely correlated to growth (Fig. 2a) and the maximum RNC remained at about 11.0 (Fig. 2b).

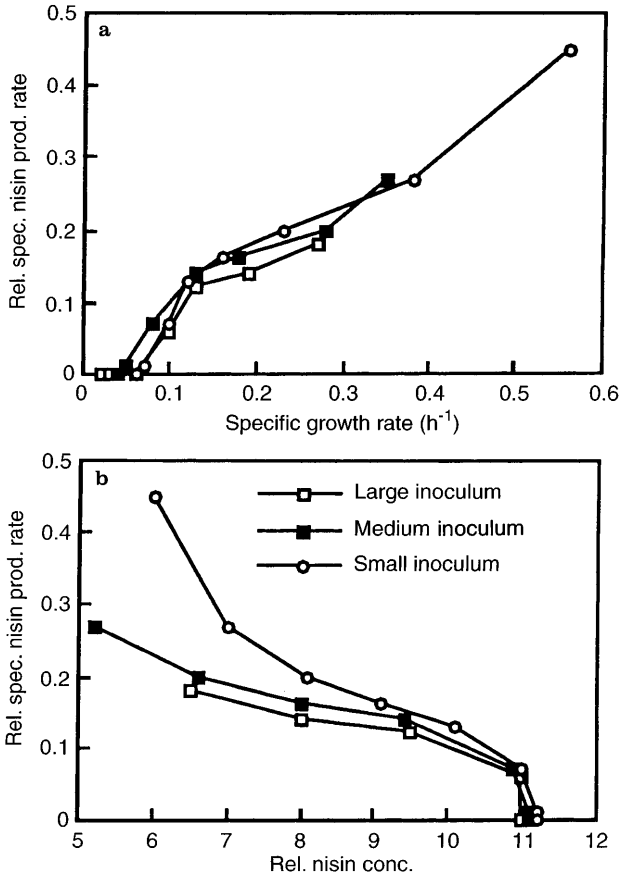

Fig. 2a,b Nisin production using different inoculum sizes: **a** the relationship between nisin production and growth; **b** nisin production at different nisin concentrations

Cultivations with added nisin

The results that have been obtained indicate that nisin production is inhibited by high concentrations of nisin. Therefore, in this study nisin was externally added to the cultivations and the effect investigated. To do this, the commercially prepared nisin, Nisaplin, was purified so as to remove any agents that might have an effect on growth or nisin production. The purified nisin was added at final RNC values of 5.0 and 11.0 at the beginning of cultivation. Growth and nisin production of C2SmPrt$^-$(Tn*Nip*) in the presence of added nisin were investigated, and the results were compared to those obtained for C2SmPrt$^-$(Tn*Nip*) with no added nisin (control). The addition of nisin exerted no significant effect on growth (data not shown). The control cultivation displayed the expected nisin-production kinetics, reaching a maximum RNC of 11.0 (Fig. 3). The cultivation to which 5.0 RNC had been added obtained its maximum RNC of 11.0 after 5 h of cultivation, and the cultivation to which 11.0 RNC had been added remained at that level for the whole course of the cultivation (Fig. 3). It therefore appears that no additional nisin was synthesized.

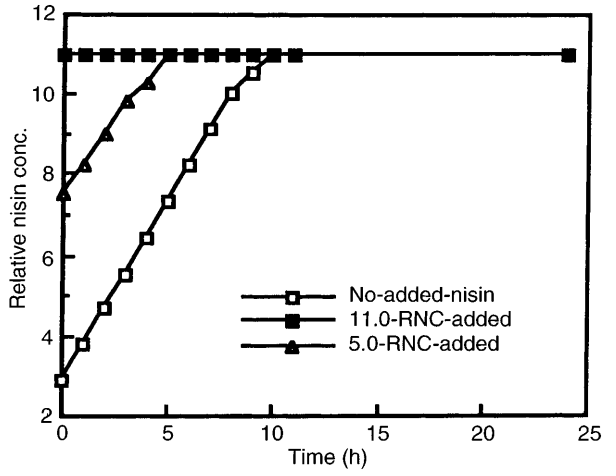

Fig. 3 Nisin production with externally added nisin and also no added nisin

Discussion

The removal of three plasmids from C2 to generate C2Prt$^-$ did not significantly affect growth, indicating that the cured plasmids are not essential for growth. The C2Prt$^-$ derived in this study showed no reduced acid production (data not shown) contrary to other Prt$^-$ variants described by Kok and Venema (1988); in fact, a slightly increased A_{max} was achieved. One trait, however, that was lost was the ability to utilize lactose. The proteinase and lactose determinants are known to be encoded on the 46-kb plasmid (McKay et al. 1976; Klaenhammer et al. 1978) that had been eliminated. When the transposon that encodes nisin production was introduced into C2SmPrt$^-$ the specific growth rate and the A_{max} reached were significantly reduced. The reduction in growth could be due to inhibition by nisin or its precursor and/or energy usage for nisin production.

Nisin production was found to be associated with growth, as demonstrated in the experiments in which the specific growth rate was altered. The energy from the excess sugar did not support any increase in production, indicating that nisin production is dependent on growth. In addition, galactose supported a much lower A_{max} compared to glucose or sucrose, and this was reflected in the lower level of nisin produced. A direct link between nisin production and growth was also demonstrated where large inoculums were used to increase the cell concentration. However, the time taken to obtain the maximum level of nisin was much shorter with larger inoculums. Detailed kinetic analyses showed that nisin production was growth-associated for a large part of growth. Nisin production occurred when the specific growth rate was greater than 0.05 h^{-1} and it ceased when the specific growth rate was 0.05 h^{-1} or less.

It was shown that the relative specific nisin production rate was not directly proportional to the specific growth rate. For example, the cultivation with low

nutrient concentration supported a relatively higher relative specific nisin production rate when compared with higher nutrient concentrations. De Vuyst et al. (1990) also demonstrated that nisin production could be increased with lower specific growth rates. The lower specific growth rates were achieved by using disaccharides as the sugar substrate instead of monosaccharides, because an apparent situation of nutrient limitation could be created with the disaccharides. Low specific growth rates arising from slow metabolism of "difficult-to-metabolize" nutrients also resulted in higher nisin production (De Vuyst and Vandamme 1993). Also, in continuous culture, the highest levels of nisin were obtained at low specific growth rates of between 0.2 h^{-1} and 0.3 h^{-1} and not at the maximum specific growth rate of 0.6 h^{-1} (Meghrous et al. 1992).

The experiment where external nisin was added demonstrated that nisin production was limited by high concentrations of nisin. When nisin was added back to the cultivations, the total level of nisin could not be raised despite the availability of excess nutrients. In the low-nutrient-concentration cultivations, nisin production was limited by nutrient depletion and not by high nisin concentrations. This was also true for the galactose cultivation, where nisin production was limited by the organism's inability to use the sugar "efficiently" and not by high nisin concentrations. Therefore, there seem to be two factors determining the nisin "ceiling" level. One is nutrient availability, which occurs when a strain grows well and quickly exhausts the available nutrient, the nisin production ceases and a "premature" ceiling is obtained. Another is nisin inhibition, which occurs when a strain grows where plenty of nutrients are available for nisin production, and production proceeds to a "real" ceiling where the high concentration of nisin inhibits further nisin production. Once the host-specific ceiling concentration of nisin is reached then nisin production stops, even if the producing strain continues to grow well.

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